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PRK2 during Meiotic Maturation of Starfish Oocytes

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The resumption of meiosis in the developing starfish oocyte is the result of intracellular signaling events initiated by 1-methyladenine stimulation. One of the earliest detectable kinase activities during meiotic maturation of starfish oocytes is a protein kinase C or PKC-like activity. In this study, several isoforms of protein kinase C were cloned from the oocyte; however, the most abundant PKC-like maternal transcript corresponds to protein kinase C-related kinase 2 (PRK2). PRK2 is expressed in the immature oocyte and at least until germinal vesicle breakdown. Subcellular localization of PRK2 revealed a cytoplasmic distribution in the immature oocyte, which, during meiotic maturation, remained in the cytoplasm but also localized to the disintegrating germinal vesicle. Significantly, PRK2 is phosphorylated *in vivo* in response to 1-methyladenine which precedes MPF activation, making PRK2 a candidate regulator of early signaling events of meiotic maturation.

INTRODUCTION

During early development, oocytes arrest late in G2 of the first meiotic cell cycle. Hormonal stimulation from surrounding follicle cells triggers a cascade of events which results in the resumption of meiosis, known as meiotic maturation (Masui and Clarke, 1979). In the case of starfish oocytes, meiotic maturation is characterized by the activation of maturation-promoting factor (MPF), which is a complex of $p34^{cdc2}$ and cyclin B; breakdown of the germinal vesicle (GVBD); and the subsequent completion of meiosis I and II with the accompanying production of polar bodies. In addition, this signaling event triggers the initiation of translation through the activation and unmasking of specific maternal mRNAs and the assembly of the translation initiation complex (Rosenthal *et al.*, 1982; Martindale and Brandhorst, 1984; Xu and Hille, 1990; Xu *et al.*, 1993).

In starfish, ''maturation-inducing hormone,'' 1-methyladenine (1MA) (Kanatani *et al.*, 1969), interacts with an oocyte surface receptor (Kanatani and Hiramoto, 1970) which initiates intracellular signaling through the activation of the $\beta\gamma$ subunit of heterotrimeric G proteins (Chiba *et al.*, 1993; Jaffe *et al.*, 1993) and, through a series of unknown signaling events, results in the activation of MPF and translation initiation. In *Xenopus* oocytes, a key mediator of progesterone-induced meiotic maturation is the serine/threonine protein kinase, Mos, the synthesis of which is required for meiotic maturation and MPF activation (reviewed in Sagata, 1997). Mos activity results in the activation of the MAP kinase pathway which again is critical for meiotic maturation in *Xenopus* oocytes (Haccard *et al.*, 1995; Kosako *et al.*, 1994). In starfish oocytes, however, no equivalent of Mos has been identified (or indeed in invertebrates to date) and thus the regulatory molecules responsible for meiotic maturation remain to be identified.

Previously Xu *et al.* (1993) showed that a protein kinase C (PKC)-like activity is detected in oocytes after 1MA treatment, and this activity precedes MPF activation and translation initiation, making PKC-like kinases potential candidates controlling the early signaling events of meiotic maturation. PKC comprises a family of proteins, some of which are regulated by diacylglycerol and calcium ions (reviewed in Dekker *et al.*, 1995). Phorbol esters are not able to induce meiotic maturation of starfish oocytes (Kishimoto *et al.*, 1985; Xu *et al.*, 1993); however, the atypical PKC isoforms, which are not regulated by such lipids, may be able to participate in meiotic maturation. Indeed, in *Xenopus*, PKCζ is involved in insulin-stimulated oocyte maturation (Dominguez *et al.*, 1992), and activation of PKC in *Chaetopterus*

The sequence for the *P. ochraceus* PRK2 gene is available in the Genbank/EMBL data base under the Accession No. AF035554 and on WWW under Banklt 150080.

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oocytes results in germinal vesicle breakdown (Eckberg *et al.*, 1996).

A preliminary approach to understanding further the contribution made by PKC or PKC-like kinases in oocyte maturation was to clone PKC family members from the starfish oocyte. The predominant clone identified, however, most closely resembled mammalian PRK2 (protein kinase C-related kinase 2) (Palmer *et al.*, 1995a). PRK2, together with its human paralogue, PRK1/PKN (Mukai and Ono, 1994; Palmer *et al.*, 1995a), was previously identified using a PCR strategy and they are thought to mediate some of the effects of the Rho family of GTPases (reviewed in Nagata and Hall, 1996). Rho family members participate in such diverse biological processes as cell cycle progression (Olson *et al.*, 1995), cytokinesis (Drechsel *et al.*, 1997), actin cytoskeleton reorganization (Ridley and Hall, 1992; Ridley *et al.*, 1992), and serum response factor-mediated transcriptional activation (Hill *et al.*, 1995). PRK1/PKN specifically interacts with Rho-GTP, resulting in an increase in PRK1/PKN kinase activity (Amano *et al.*, 1996; Watanabe *et al.*, 1996), while PRK2 can bind to both Rho and Rac GTPases (Vincent and Settleman, 1997). Further protein–protein interaction analyses have shown PRK1/PKN capable of binding and phosphorylating structural intermediate filament proteins (Matsuzawa *et al.*, 1997) and α-actinin (Mukai *et al.*, 1997), while PRK2 can bind the adaptor protein NCK (Quilliam *et al.*, 1997). Thus, these evolutionarily conserved kinases, PRK1/PKN and PRK2, are likely to participate in a variety of cellular signaling events.

Here we present the identification of starfish PRK2 as the major PKC-like kinase transcript in the oocyte. Specific polyclonal antibodies show that PRK2 protein is expressed at constant levels in the immature oocyte and at least until the stage of GVBD. Phosphorylation of PRK2 occurs very early during the resumption of meiosis and prior to the activation of MPF. Furthermore, immunolocalization studies indicate that PRK2 is in the cytoplasm of the oocyte which, during meiotic maturation, appears to localize to the germinal vesicle. The possible roles for PRK2 during meiotic maturation are discussed.

MATERIALS AND METHODS

Animals and Oocyte Culture

Starfish, *Pisaster ochraceus,* were collected from the Olympic Peninsula, Washington, and maintained in aerated natural seawater before use. Oocytes were isolated from ovaries, washed in several changes of calcium-free seawater (400 mM NaCl, 10 mM KCl, 10 mM Tris, pH 8.0, 0.5 mM EGTA, 10 mM $MgCl_2$, 29 mM $MgSO_4$) to remove follicle cells, and resuspended at 5% (v/v) in artificial seawater (400 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 20 mM $MgCl_2$, 29 mM $MgSO_4$, 10 mM Hepes, pH 8.0) for subsequent procedures. Oocytes were matured at 15°C *in vitro* by the addition of 2 μM 1-methyladenine (Sigma); germinal vesicle breakdown occurred approximately 50 min later.

PCR Cloning of PKC Isoforms

Redundant oligonucleotides were designed within subregions V and VIII of the conserved catalytic domain of PKC (5′-AAC/T GGI GGI GAC/T C/TTI ATG-3′ and 5′-GTA GTC IGG A/GGT ICC A/GCA A/GAA-3′) (Hanks *et al.*, 1988). Through PCR on first-strand cDNA prepared from oocyte and gastrula mRNA, the primers produced 220- to 240-bp products. The PCR fragments were purified and cloned into the TA vector (Invitrogen). Resulting clones were sequenced using the Sequenase 2.0 kit (USB) and identified by comparison of sequences contained in the GenBank/EMBL databases. The strategy used to clone Rho will be published elsewhere.

cDNA Libraries and Cloning

Oocyte- and gastrula-stage oligo(dT)-primed cDNA libraries were prepared from poly(A)-selected RNA using oligo(dT) cellulose. First-strand cDNA was prepared using an oligo(dT) primer and Superscript reverse transcriptase (Gibco BRL); second strand was synthesized using RNaseH, DNA polymerase I, and *Escherichia coli* DNA ligase. Following ligation of *Eco*RI–*Not*I adaptors, the cDNA was inserted into the *Eco*RI site of Lambda ZAP II bacteriophage vector (Stratagene). The library was screened with the radiolabeled PCR fragment corresponding to PRK2 (Prime-It II; Stratagene). Hybridization was carried out at 68°C in 0.25 M $NaPO_4$, pH 7.2, 7% SDS, 1 mM EDTA, 1% BSA. Filters were washed (three times, 20 mM $NaPO_4$, pH 7.2, 1% SDS, 1 mM EDTA, 20 min, 68°C) and exposed for autoradiography. cDNA clones were fully sequenced by creating exonuclease III deletions (Erase-a-Base; Promega). Sequences were analyzed using the University of Wisconsin Genetics Computer Group Package, Version 8.1.

Northern Analysis

Total RNA was prepared from oocyte, blastula, and gastrula stages of development by homogenization in 80 mM KCl, 100 mM KGluconate, 25 mM Hepes, 2 mM EGTA, 2.5 mM $MgCl_2$, 300 mM glycine, 230 mM glycerol followed by acid phenol extractions and ethanol precipitation. Electrophoresis of 10 μg of total RNA in a 1% agarose, 200 mM Mops, 7% formaldehyde gel was followed by capillary transfer to a Hybond-N nylon filter (Amersham) and baking for 2 h at 80°C. Hybridization of PKC- or PRK2-specific radiolabeled DNA probes was performed as described above.

Antibodies

PRK2 polyclonal antibodies were produced in rabbits (R&R Rabbitry, Stanwood, WA). Antiserum 2140 was prepared using a synthetic peptide from the C-terminal of starfish PRK2 (CGGQEQNLFKDFNYTADW) conjugated to keyhole limpet hemocyanin. Antiserum 2298 was prepared using a bacterially produced glutathione *S*-transferase (GST) fusion protein containing an N-terminal fragment of PRK2 (indicated in Fig. 3b; equivalent amino acids in human PRK2 are 39–153). Antiserum 2298 was partially purified using a GST–PRK2 fusion protein-coupled column (prepared using Pierce AminoLink immobilization kit). Rabbit polyclonal cdc2 (PSTAIR) antibody was a gift from S. Pelech. Starfish cyclin B antibody was a gift from T. Kishimoto (Ookata *et al.*, 1992).

Protein Extracts and Western Analysis

Oocyte protein extracts were prepared by freeze/thawing oocytes in lysis buffer (60 mM β-glycerol phosphate, 30 mM *p*-nitrophenyl phosphate, 25 mM Mops, pH 7.2, 15 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, 0.1 mM sodium vanadate, 1 mM PMSF, and 15 μg/ml each aprotinin and leupeptin) and centrifugation at 8800*g* to recover the supernatant. Western blots were prepared essentially as described previously (Xu *et al.*, 1993). Positive signals on immunoblots were detected using rabbit αIgG-horseradish peroxidase and ECL (Amersham) according to instructions provided.

In Vivo Labeling of Oocytes and Immunoprecipitation

Oocytes were suspended in artificial seawater and incubated at 15°C for 1 h in the presence of 0.2 mCi/ml *o*-[^{32}P]phosphate (8500 Ci/mmol; New England Nuclear). The oocytes were washed extensively in artificial seawater and cultured at 15°C. Then 2 μM 1-methyladenine was added and samples were taken at successive time points for preparation of protein extracts as described above. To immunoprecipitate PRK2 from extracts, 2 μl anti-PRK2 N-terminal antibody (2298) was absorbed to protein A–Sepharose (Bio-Rad) in IP buffer (10 mM Hepes, pH 8.0, 1 mM EDTA, 10% glycerol, 50 mM NaCl, 2 mM DTT, 0.1% Triton X-100) for 2 h at 4°C. Protein extracts were precleared with the same beads in IP buffer for 1 h at 4°C. The antibody–protein A–Sepharose was washed once in IP buffer and the precleared lysate was added and incubated for 2 h at 4°C. The Sepharose was washed three times in IP buffer containing 0.5 M NaCl, then resuspended in SDS–PAGE sample buffer and boiled for 5 min.

Whole-Mount Immunocytochemistry and Image Analysis

Immature oocytes were first treated with 0.1 mg/ml Pronase (Sigma) in artificial seawater at 4°C for 10 min to remove jelly coat and vitelline layers. Oocytes were washed extensively in calcium-free seawater before being resuspended in artificial seawater and cultured. Oocytes were fixed (3.6% EM grade formaldehyde, 0.65 M glycerol, 4 mM EDTA, 0.72 mM Tame, 72 mM Pipes, pH 7.0) overnight at 4°C. This was followed by blocking in 5% BSA or heat-inactivated goat serum, 0.3% Triton X-100 in PBS overnight. Oocytes were incubated in anti-PRK2 (2298) antibody (1:50) in blocking solution overnight at 4°C, then washed extensively in PBS/0.3% Triton X-100. The oocytes were further incubated in FITC-conjugated goat anti-rabbit IgG (Cappel) (1:100 in blocking solution) overnight at 4°C, followed by extensive washing, and were finally mounted in PBS containing 20% glycerol and 100 μg/ml DABCO (Sigma). Immunostained oocytes were analyzed using a Bio-Rad MRC-600 scanning confocal microscope and COMOS software (Bio-Rad).

RESULTS

PRK2 and Protein Kinase C Isoforms Present in the Starfish Oocyte

To further characterize the PKC activity previously observed in oocytes after 1-methyladenine treatment (Xu *et al.*, 1993), redundant PCR primers were used to clone PKC isoforms from starfish oocytes. Primers were designed within subdomain V of the catalytic domain (Hanks *et al.*, 1988), which is highly conserved between PKC isoforms, and to subdomain VIII, which is conserved in all serine/threonine kinases. cDNA was synthesized from both oocyte and gastrula mRNA, and PCR products of the predicted size were cloned (Fig. 1). Each 220- to 240-bp clone was sequenced and then compared to sequence databases for identification. Clones of identical sequence were grouped according to which PKC member they most resembled. By far, the predominant clone identified corresponded to a protein kinase C-related kinase, PRK2 (Palmer *et al.*, 1995a) (Fig. 1). From oocyte mRNA, 32 of 37 clones most closely resembled PRK2. PCR products most similar to PKCα and PKCϵ also were cloned from oocyte, representing 11 and 3% of total clones, respectively. Although fewer clones were characterized from gastrula mRNA, PCR clones representing PRK2 were again most abundant (50%); 20% of clones corresponded to the PKCα and 10% to the PKCζ isoform. Interestingly, these primers also yielded from gastrula two clones of a non-PKC kinase, cGMP-protein kinase (data not shown), which is phylogenetically most closely related to the PKC family (Hanks *et al.*, 1988). The catalytic domain of PRK2 (and its mammalian homologue PRK1/PKN) is highly homologous to PKC and thus, with the PCR cloning strategy adopted here, it is not surprising that PRK2 was cloned.

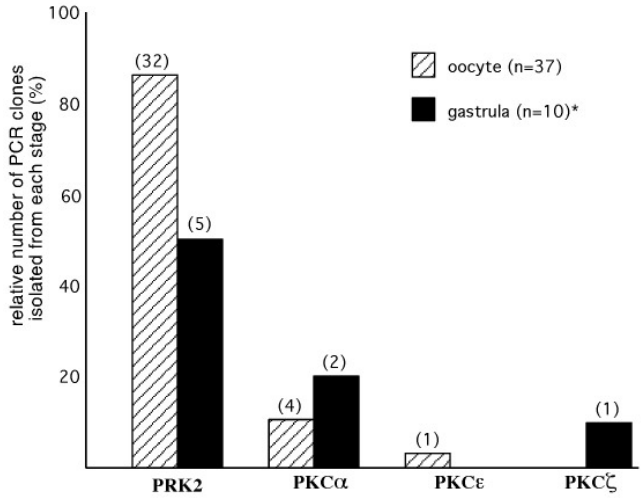

FIG. 1. PCR cloning of protein kinase C isoforms from starfish oocyte and gastrula stages. PCR products (200–220 bp), produced using redundant primers from within the catalytic domain of protein kinase C, were identified by comparison to database sequences. Actual numbers of clones are indicated above each column. (*Another two clones, corresponding to cGMP-dependent protein kinase, were also cloned from gastrula but not included in the graph.)

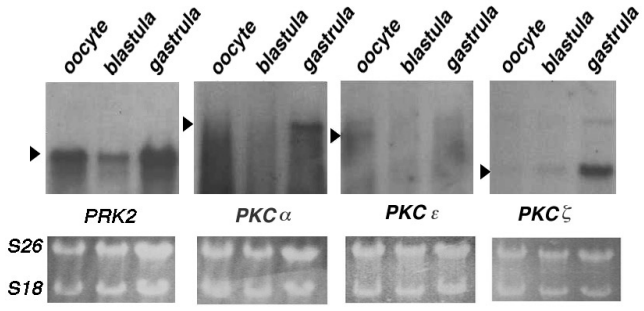

FIG. 2. Northern analysis of PKC isoforms and PRK2 in starfish oocytes and postfertilization embryonic stages. 10 μg of total RNA from oocyte, blastula, and gastrula stages was used to prepare Northern blots. PCR products of PKCα, ϵ, and ζ and PRK2 were radiolabeled and used as probes. Autoradiographic exposure time for each Northern was 24 h with an intensifier screen, except for PKCϵ, which was exposed for 96 h. Relatively equivalent amounts of RNA are in each sample, as indicated by ethidium bromide staining of rRNA subunits.

PRK2 Represents the Major PKC-like Maternal Transcript in Oocytes

Northern analysis was used to determine the relative expression levels of PRK2 and the PKC transcripts in the oocyte. While the previous PCR results suggested that PRK2 was the most abundant PKC-like maternal transcript in the oocyte, this may have been due to a bias in the primers or an artifact of cloning. Total RNA from immature oocytes and embryonic/postfertilization blastula and gastrula stages was probed with the PCR products for PRK2 and each PKC clone. PRK2 was highly expressed as an 8-kb transcript in all stages of starfish development examined (Fig. 2). In contrast, the PKC isoforms varied in levels and stages of expression. The PKCα probe recognized a 10-kb transcript present in the oocyte and at much greater levels in gastrulae. The PKCϵ probe only barely detected an 8-kb transcript at all stages. The 5.6-kb PKCζ mRNA was expressed most significantly much later in development, although transcripts for PKCζ do exist in the immature oocyte at very low levels, as a larger cDNA clone for PKCζ has been isolated from the oocyte cDNA library (unpublished, G.S.). A faint 10-kb transcript also is detected with both the PKCϵ and the PKCζ probes; however, because these probes are very similar to PKCα in the catalytic domain, it is likely that the 10-kb bands are the result of cross-hybridization with PKCα.

Starfish PRK2

In order to obtain larger cDNA clones encoding starfish PRK2, cDNA libraries were constructed from both oocytes and gastrulae. The oocyte library was screened with the radiolabeled PRK2 PCR product, resulting in nine positive clones (Fig. 3a). Clone 6.2, 3.98 kb in length, contained the open reading frame for PRK2, excluding approximately 38 amino acids from the amino terminal (based on comparison with mammalian PRK2) (Fig. 3b). Clone 4.5 overlaps 6.2 and extends through a 2.96-kb 3′ untranslated region to an ATTAAA polyadenylation signal located 20 nucleotides before a poly(A) tail (data not shown). Further screening of the libraries failed to isolate the remaining N-terminal PRK2 sequence.

The open reading frame of starfish clone 6.2 encodes a serine/threonine kinase which is 77% homologous to human PRK2 (Fig. 3b). Clone 6.2 contains all previously identified domains in human PRK2. Homology region 1 (HR1a, b, and c) domains which each contain a leucine heptad sequence are 67, 64, and 52% identical, respectively, to human PRK2. Sequences identified in human PRK1/PKN as a pseudosubstrate site (amino acids 39–53 in human PRK1/PKN) (Kitagawa *et al.*, 1996) are conserved, as are the sequences responsible for Rho binding (amino acids 33–111 in human PRK1/PKN) (Shibata *et al.*, 1996). Homology region 2 (HR2), which is conserved in all PRKs, is 77% identical to human PRK2. The C-terminal catalytic domain is highly homologous to the protein kinase C family and is 87% identical to human PRK2.

PRK2 Antibodies Detect a 130-kDa Protein in Immature Oocytes and throughout Meiotic Maturation

We chose to investigate further the role of PRK2 during oocyte meiotic maturation for a number of reasons. First, PKCα and PKCϵ, both of which require diacylglycerol/phorbol ester for activation, were no longer considered, as phorbol ester is inhibitory to meiotic maturation of starfish oocytes (Kishimoto *et al.*, 1985; Xu *et al.*, 1993). Second, although it is still possible that PKCζ could be responsible for the observed PKC-like activity, based on transcript abundance, PRK2 is the most prevalent PKC-like kinase in the oocyte and so most likely to fulfill this function.

Unlike *Xenopus* meiotic maturation, protein synthesis is not required for meiosis I in starfish oocytes; therefore, early regulatory events during meiotic maturation must be controlled by preexisting proteins in the immature oocyte. Therefore, to examine the role of PRK2 in starfish oocytes, polyclonal antibodies were raised against two different regions of the PRK2 protein (Fig. 4a). The N-terminal 2298 antiserum and the C-terminal 2140 antiserum both recognize a 130-kDa protein in immature oocytes (Fig. 4a). A second prominent 80-kDa band is also recognized by N-terminal antiserum 2298, which may be the result either of degradation or of cross-reactivity. In oocytes which have undergone meiotic maturation, both anti-PRK2 antisera detected a slight decrease in the electrophoretic mobility of PRK2 (Fig. 4a, lanes I and GVBD). To determine the precise time at which this shift in PRK2 mobility occurs, Western analysis using anti-PRK2 antiserum 2298 was performed on lysates prepared from oocytes at various stages of meiotic

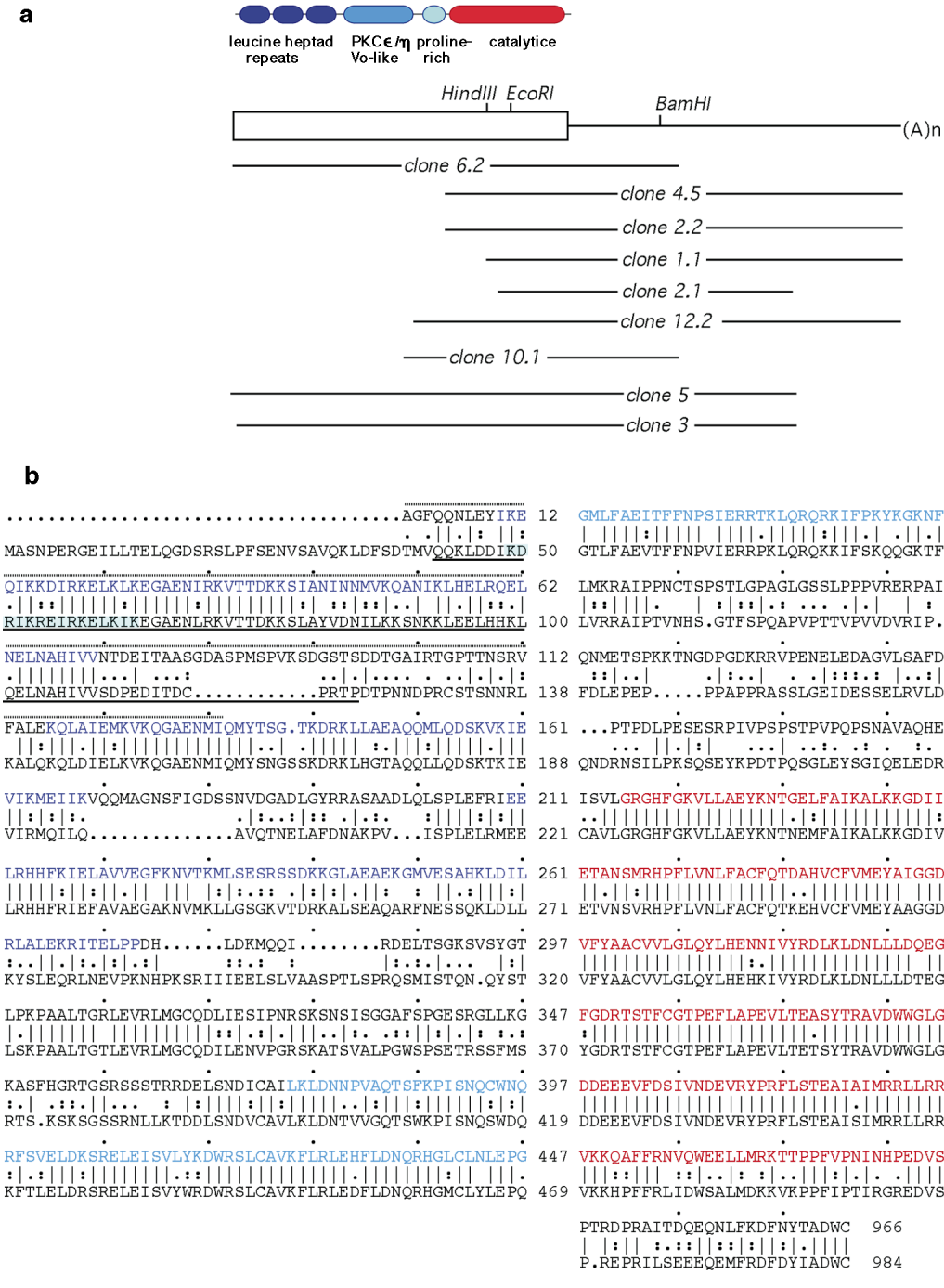

FIG. 3. Starfish PRK2. (a) PRK2 cDNA clones isolated. The nine clones were aligned by sequence and restriction site mapping. (b) Starfish PRK2 (top) amino acid sequence and alignment with human PRK2 (bottom). Broken line indicates PRK2 sequence fused to glutathione *S*-transferase used for antibody production; black line indicates corresponding PRK2 sequence analogous to Rho-binding domain in PRK1/PKN; blue-boxed sequence (amino acids 49–63) indicates corresponding PRK2 sequence equivalent to pseudosubstrate in PRK1/PKN. HR1a, b, and c sequences are dark blue; HR2 sequence is light blue; catalytic domain sequence is red.

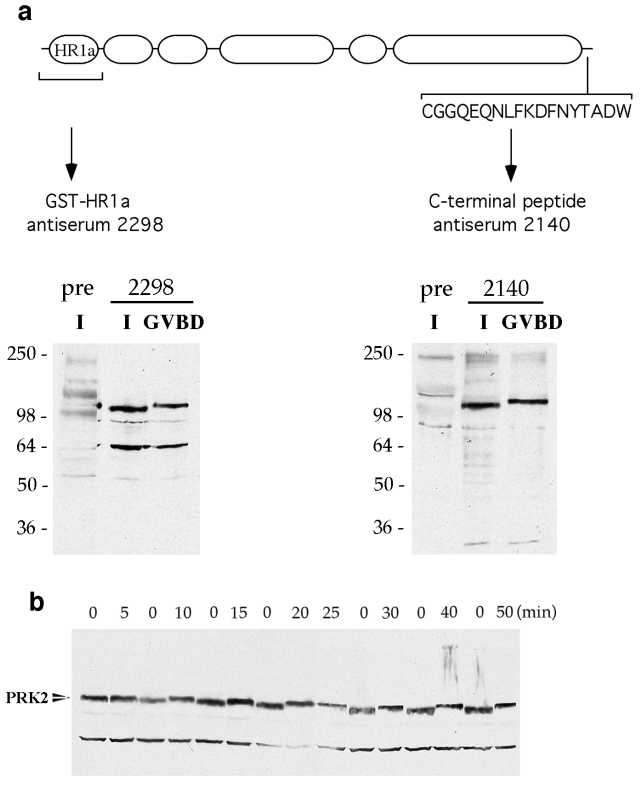

FIG. 4. PRK2 antibodies and Western analysis. (a) Antibody design and Western analysis of PRK2 from starfish oocytes. Total protein extracts from immature (I) and oocytes at GVBD stage (GVBD) were analyzed on an 8% SDS–PAGE gel. Western blots were probed with preimmune serum (pre) or anti-PRK2 antibodies (2298 and 2140). (b) Western analysis of PRK2 during meiotic maturation of starfish oocytes. Total protein extracts from oocytes were prepared at various time points after addition of 1-methyladenine and run on an 8% SDS–PAGE gel. The Western blot was probed with antiserum 2298. 1-Methyladenine was added at time 0; GVBD occurred at 50 min. Multiple 0 time-point samples were included in order to detect more precisely the shift in PRK2 mobility.

maturation (Fig. 4b). Several protein samples from immature oocytes (0 time point) were included in the Western to precisely determine when the shift in PRK2 occurred. Ten minutes after the addition of 1-methyladenine, all detectable PRK2 shifted to the slower migrating form which remained for the rest of the time course, at least until GVBD. Such shifts in electrophoretic mobility of a protein often correlate with a change in the phosphorylation state.

PRK2 Is Phosphorylated in Vivo during Meiotic Maturation Prior to p34^{cdc2} Dephosphorylation

PRK1/PKN and PRK2 have both been shown to autophosphorylate *in vitro,* which correlates with *in vitro* kinase activity (Palmer *et al.,* 1995a; Peng *et al.,* 1996; Yu *et al.,* 1997). In order to determine whether PRK2 is activated during meiotic maturation of oocytes and whether the observed shift in electrophoretic mobility is due to phosphorylation, we examined the phosphorylation of PRK2 *in vivo* after 1-methyladenine treatment. Oocytes were cultured in the presence of radiolabeled *o*-phosphate for 1 h prior to the addition of 1-methyladenine. PRK2 was immunoprecipitated from protein extracts prepared at different stages during meiotic maturation and analyzed by SDS–PAGE, Western blotting, and autoradiography.

There is no detectable phosphorylation of PRK2 in the immature oocyte; however, within minutes of 1-methyladenine addition, PRK2 was phosphorylated (Fig. 5a, top). PRK2 phosphorylation was detected by 10 min after the resumption of meiosis and reached maximal levels around 30 min (Figs. 5a and 5c). Two other phosphorylated bands which do not immunoprecipitate with preimmune serum were also detected using the anti-PRK2 2298 antibody: a 110-kDa band appeared at 10 min and disappeared after 25 min, while the upper 180-kDa band appeared at 15 min after 1-methyladenine addition. After autoradiography, the filter was probed with anti-PRK2 antibody which recognized a single 130-kDa protein in each lane (Fig. 5a, bottom). In contrast, the change in cdc2 electrophoretic mobility, which correlates with changes in cdc2 dephosphorylation and H1 kinase activity (Xu *et al.,* 1993), did not occur until 20 min after treatment with 1-methyladenine (Fig. 5b). Thus PRK2 phosphorylation occurs very early in the maturing oocyte and precedes the activation of MPF.

Subcellular Localization of PRK2 in the Immature Oocyte and during Meiotic Maturation

The subcellular distribution of PRK2 in the oocyte during meiotic maturation was investigated in order to gain further insight into the possible location of PRK2 activity and regulation. In the immature oocyte, when PRK2 is in an inactive state, PRK2 protein is distributed throughout the cytoplasm, but is excluded from the germinal vesicle (Fig. 6A). Various controls, including staining of oocytes with the secondary FITC-labeled antibody alone, which gave no signal, and staining with cyclin B antibody, which gave the correct expression pattern activity (Ookata *et al.,* 1992), were used to confirm the use of the fixation and staining methods used (data not shown). The localization of PRK2 protein was next examined at various stages after 1-methyladenine treatment. At 20 min after hormone treatment, when PRK2 phosphorylation has occurred (see Figs. 4b and 5a), PRK2 immunostaining is detected in the germinal vesicle as well as in the cytoplasm (Fig. 6B). At 40 min, when the germinal vesicle is disintegrating, PRK2 remains partially localized to the germinal vesicle and the cytoplasm (Fig. 6C). By 60 min, as GVBD is completed, phosphorylated PRK2 is evenly distributed throughout the cytoplasm of the mature oocyte (Fig. 6D). PRK2 phosphorylation is maintained at least until this stage.

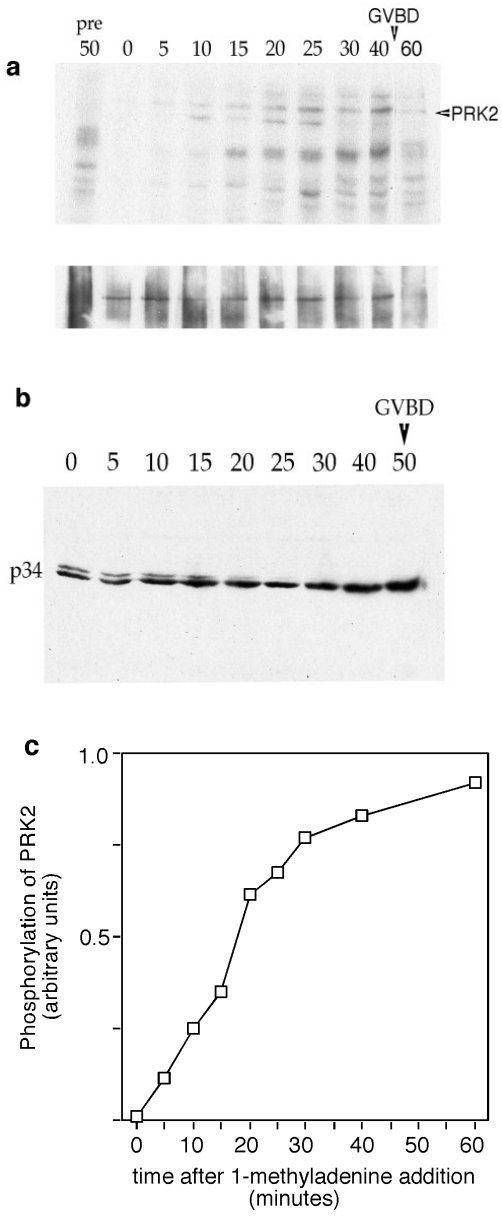

FIG. 5. PRK2 activation in starfish oocytes during meiotic maturation. (a) Time course of PRK2 autophosphorylation *in vivo* after addition of 1-methyladenine. Oocytes were incubated in the presence of *o*-[^{32}P]phosphate for 1 h prior to the addition of 1-methyladenine. Protein lysates were made at the times indicated, and PRK2 was immunoprecipitated using anti-PRK2 antibody 2298 [or preimmune (pre) serum]. Samples were run on an 8% SDS–PAGE gel, blotted, and exposed for autoradiography (top). PRK2 was identified by probing the same blot with anti-PRK2 antiserum 2298 (bottom). (Note that the conditions used for SDS–PAGE in this particular experiment did not resolve the difference in electrophoretic mobility of PRK2 well.) (b) Change of p34^{cdc2} electrophoretic mobility during time course of meiotic maturation. Oocyte protein extracts were analyzed on a 12% SDS–PAGE gel, blotted, and probed with rabbit polyclonal anti-PSTAIR antibody. (c) Quantitation of PRK2 autophosphorylation. The relative incorporation of phosphate into PRK2 was measured by densitometry using the autoradiogram in a and quantified using the Molecular Image System (Bio-Rad) and NIH Image.

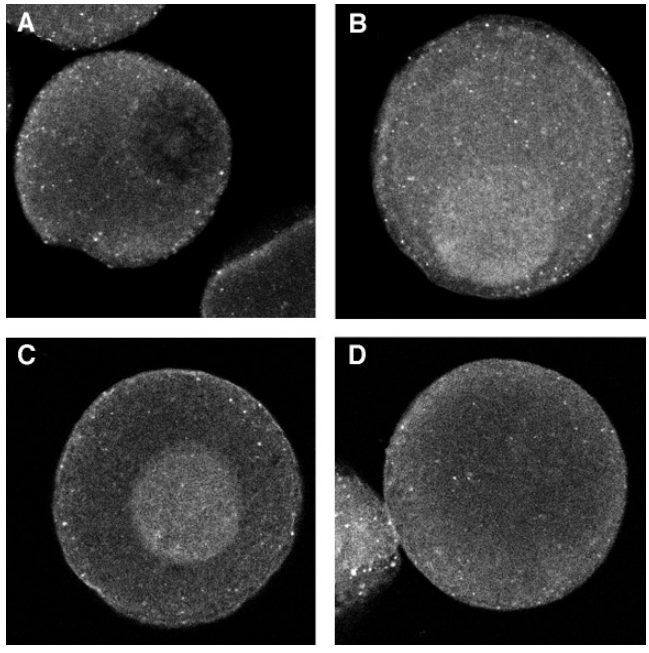

FIG. 6. Time course of PRK2 localization during meiotic maturation of starfish oocytes. At times indicated, oocytes were fixed then stained with anti-PRK2 antibody (2298). (A) Immature oocyte, (B) 20 min, (C) 40 min, and (D) 60 min after 1-methyladenine addition.

Rho, a Potential Regulator of PRK2, Is Highly Expressed in Starfish Oocytes

Rho family members have been shown to regulate PRK1/PKN and PRK2 activity (Amano *et al.*, 1996; Watanabe *et al.*, 1996; Vincent and Settleman, 1997). Commercially available antibodies to mammalian Rho were unable to detect Rho in starfish protein extracts (data not shown). Given the high degree of similarity to Rho from human and another marine invertebrate, *Aplysia,* we used redundant oligonucleotides and PCR to clone Rho from starfish oocytes. Two overlapping fragments of Rho that span from the effector domain to the Rho family insert (amino acids 30–139 in human RhoA) were cloned from starfish oocyte cDNA (Fig. 7a). Across this region, starfish Rho is highly similar to human RhoA, with only six amino acid changes. These fragments were then used as probes to examine Rho transcript levels in the oocyte (Fig. 7b). Northern analysis of poly(A)$^+$-selected RNA from oocytes and gastrula embryos revealed an abundant single 2.7-kb transcript recognized by both Rho cDNA fragments.

a

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human RhoA          MAAIRKKLVI  VGDGACGKTC  LLIVFSKDQF  PEVYVPTVFE  NYVADIEVDG
Aplysia Rho         ----------  ----------  ----------  ----------  ----------
Pisaster clone 22                                   -  ----------  ----------

human RhoA          KQVELALWDT  AGQEDYDRLR  PLSYPDTDVI  LMCFSIDSPD  SLENIPEKWT
Aplysia Rho         ----------  ----------  ----------  ----------  ----------
Pisaster clone 22   -C--------  ----------  ----------  -------N-
Pisaster clone 11              -------     ----------  -------N--  ----------

human RhoA          PEVKHFCPNV  PIILVGNKKD  LRNDEHTRRE  LAKMKQEPVK  PEEGRDMANR
Aplysia Rho         ---R------  ----------  -----S-K--  -M-------R  --D--A--EK
Pisaster clone 11   ----------  -V--------  -----A-K--  -Q-------

human RhoA          IGAFGYMECS  AKTKDGVREV  FEMATRAALQ  ARRGKKKSGC  LVL
Aplysia Rho         -N-YS-L---  ----E---D-  --T-------  VKK.---G--  V--
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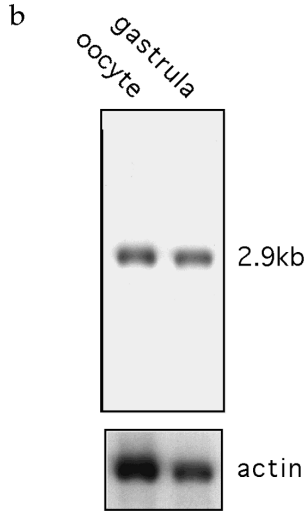

FIG. 7. Cloning and expression of Rho in the starfish immature oocyte. (a) Amino acid sequence alignment of starfish clones with *Aplysia* and human RhoA. (b) Northern analysis of starfish Rho. 1 μg of poly(A)$^+$-selected RNA from oocyte and gastrula was probed with radiolabeled starfish PCR clone 11. Autoradiographic exposure time was 15 h with intensifier screens.

DISCUSSION

During the reinitiation of meiosis, a number of kinase activities are regulated in response to hormonal stimulation, including stimulation of PKC-like activity (Xu *et al.*, 1993), myelin basic protein kinase activity (Pelech *et al.*, 1988), and tyrosine kinases (Peaucellier *et al.*, 1990). In the present study, we have established that the protein kinase C-related kinase, PRK2, is the major maternal PKC-like kinase in the immature oocyte. Prior to meiotic maturation, PRK2 is in the cytoplasm of the oocyte, unphosphorylated and presumably inactive. Exposure of oocytes to 1-methyladenine induces meiotic maturation along with an increase in phosphorylation of PRK2, which, from previous work, correlates with PRK2 activity (Palmer *et al.*, 1995a; Yu *et al.*, 1997). Associated with PRK2 phosphorylation, PRK2 protein moves into the germinal vesicle during meiotic maturation. It is most likely that PRK2 accounts for the PKC activity previously described in oocytes, even though PRK2 phosphorylation occurs within 10 min of 1-methyladenine treatment while PKC activity previously was detected at 15 min (Xu *et al.*, 1993).

While much less abundant than PRK2, a single representative of each of the ''classical'' (PKCα), ''novel'' (PKCϵ), and ''atypical'' (PKCζ) transcripts of PKC is present in starfish oocytes. In mammals the PRK family has at least three

members; however, it seems likely that again only one isoform of the PRK family is represented in starfish, as the PCR cloning approach should have been able to detect additional family members. Cloning of PRK1/PKN has been reported in *Drosophila* (Ueno *et al.*, 1997), although that paper describes only the catalytic domain and not the full clone, so it is possible that that clone may be PRK2. We believe that clone 6.2 encodes PRK2 and not PRK1/PKN as, although the open reading frame is only slightly more similar to PRK2 than to PRK1/PKN, each individual domain is significantly more similar to PRK2 than to PRK1/PKN.

Because PRK2 activity is detected before the activation of $p34^{cdc2}$, it is possible that PRK2 provides an early link between receptor-mediated meiotic maturation and activation of MPF. Similarly, an increase in a PKC-like activity precedes initiation of translation and phosphorylation of translation initiation factor, eIF-4e (Xu *et al.*, 1993). A protein kinase C isoform may phosphorylate eIF-4e in mammals (Whalen *et al.*, 1996); however, because substrate specificities of PKCs and PRKs overlap (Mukai *et al.*, 1994), it is quite possible that in the starfish oocyte, PRK2 may be directly responsible for eIF-4e phosphorylation during initiation of translation. Phosphorylation of starfish eIF-4e by PRK2 is currently being addressed.

The regulation of PRK2 activity in the oocyte awaits further investigation. PRK1/PKN and PRK2 are activated by various lipids, including arachidonic acid (Mukai *et al.*, 1994; Yu *et al.*, 1997) and phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate (Palmer *et al.*, 1995b), and by small G proteins. Interestingly, arachidonic acid is able to induce meiotic maturation in some species of starfish oocytes and *Xenopus* oocytes (Meijer *et al.*, 1984; Carnero and Lacal, 1993). We have so far, however, been unable to induce meiotic maturation with arachidonic acid in *Pisaster* oocytes either by external application or by microinjection (G.S. and M.B.H., unpublished results). Activation of PRK2 may require an additional event such as redistribution or binding of other regulatory proteins. The PRK2 distribution described in oocytes is similar to that described by Mukai *et al.* (1996), in which certain stress conditions induce translocation of PRK1/PKN from a cytoplasmic location into the nucleus of cultured fibroblasts. While it is suggested that this translocation to the nucleus may reflect PRK1/PKN regulation of transcriptional activity, it is clear that in the oocyte, PRK2 is not involved in the regulation of transcription which is activated zygotically.

A number of Rho effector kinases have been cloned, among which are PRK1/PKN and PRK2 (Nagata and Hall, 1996). It is yet to be established whether PRK2 is regulated by a small G protein in the developing oocyte, and while Rho maternal transcripts are highly abundant in the oocyte, Rho protein has not been detected so far. Equally, Rac may be responsible for regulation of PRK2 activity. Given the well-documented role of Rho family members in the regulation of the actin cytoskeleton, a predicted function of PRK2 may be the regulation of the actin-based cytoskeleton in the oocyte. Indeed, Vincent and Settleman (1997) have provided

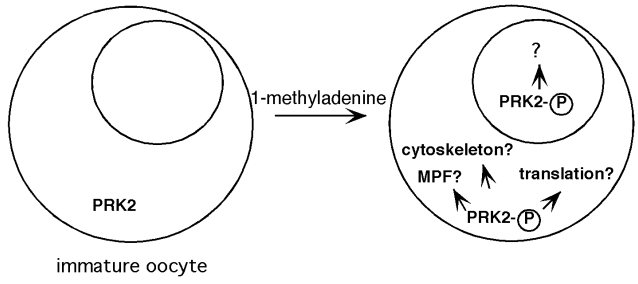

FIG. 8. Potential roles for PRK2 in starfish oocytes. 1-Methyladenine-induced meiotic maturation of starfish oocytes results in the rapid phosphorylation of PRK2 in the cytoplasm, after which, phosphorylated PRK2 partially relocates to the germinal vesicle. Possible cellular events which may lie downstream of PRK2 such as MPF activation, translation initiation, and regulation of the cytoskeleton are proposed.

preliminary evidence that PRK2 is involved in the formation of actin stress fibers in cultured cells; however, in another study, Amano *et al.* (1997) refer to unpublished data demonstrating that a constitutively active form of PRK1/PKN was unable to induce stress fibers and focal adhesions in serum-starved Swiss 3T3 cells. Changes in the oocyte actin cytoskeleton during meiotic maturation have been well characterized (Heil-Chapdelaine and Otto, 1996; Schroeder and Stricker, 1983) and will provide a basis for possible PRK2 involvement in regulation of the oocyte cytoskeleton. Additionally, toxins produced in *Clostridium* species which inactivate Rho GTPases will be used to examine the role of Rho and PRK2 in the oocyte.

While the early phosphorylation of PRK2 suggests its role as a regulator of early events during meiotic maturation, PRK2 phosphorylation is sustained at least until after GVBD, which suggests additional roles for the kinase (see Fig. 8). PRK2 phosphorylation most likely occurs in the cytoplasm before its entry into the germinal vesicle. Thus, the cytoplasmic activation of the $p34^{cdc2}$-cyclin B (Ookata *et al.*, 1992) and translation initiation and cytoskeletal changes are all potential targets of PRK2 activity. Phosphorylated PRK2 is also present later in the germinal vesicle and therefore additional substrates and functions for the kinase presumably occur there. It will be interesting to determine whether, for example, PRK2 in the germinal vesicle corresponds to the germinal vesicle factor which is required for MPF activation and GVBD (Kishimoto *et al.*, 1981; Picard and Doree, 1984). The starfish oocyte provides an interesting system for further understanding PRK2 function and regulation and its involvement in oocyte development.

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